PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Praveen SHARMA et al.** Conf. No.: **8084**

Appln. No.: **10/727,576** Group Art Unit: **1634**

Filed: **December 05, 2003** Examiner: **Juliet C. Switzer**

For: **METHOD OF PREPARING A STANDARD DIAGNOSTIC GENE TRANSCRIPT PATTERN**

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner of Patents
P.O. Box. 1450
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I, Praveen Sharma, an indian citizen residing at Lille Borgen vei 1A, 0370 Oslo, Norway;

declare as follows:

1. I am an inventor on the present application and the Director of Technology and Product Development at Diagenic AS. I hold a PhD degree in the field of molecular biology.

2. The type and progression of breast cancer is characterized by physicians by staging. The stages range from stage 0 to stage IV. Stage 0 denotes the very earliest stage of breast cancer and is referred to as carcinoma *in situ*. Two types of breast cancers fall within this stage, namely ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) which are both characterized as cancers which have remained localized within the breast's ducts or lobules, i.e. have not spread beyond the ducts or lobules at which point they would become infiltrating or invasive.

3. Reference to very early stage cancer is understood in the field to refer to stage 0 cancer. Annex 4 provides references to support this statement. The document by Kinne (Cancer, February Supplement, 1991, Vol. 67, p1196-1198, document 1) details the staging convention for breast cancer. It will be noted from Table 2 on page 1197 that the earliest stage is stage 0. Frykberg *et al* (World Journal of Surgery, 1994, Vol. 18, p45-57, document 2) describes the earliest stage of breast carcinoma as the intraepithelial or noninvasive stage otherwise known as *in situ* carcinoma to denote the fact that it is a preinvasive stage (page 45, left hand column, second full paragraph). This earliest stage includes DCIS and LCIS.

4. The National Cancer Institute (NIH Publication No. 04-5515, document 3) describe DCIS and LCIS as stage 0 and refer to DCIS as very early breast cancer (see step 2 on

page 2 and the glossary on page 20 of the publication). Whilst this article indicates that LCIS is not cancer, it will be noted that it is included in the classification of stage 0 or very early breast cancers. This is confirmed in the University of Texas's article on breast cancer (document 4), where it is indicated in relation to breast cancer *in situ* (at the top of page 2) that both DCIS and LCIS are very early breast cancers which are considered breast cancer *in situ* for the purposes of classification. See also Novartis's "Ribbon of Pink" article (document 5) which describes both DCIS and LCIS as stage 0 breast cancers which are very early breast cancer (page 2). The NCI's classification is confirmed in the AHRQ's (a Public Health Service agency in the US Department of Health and Human Services) website article, document 6, see step 2 on page 2. The National Comprehensive Cancer Network and American Cancer Society's Breast Cancer Treatment Guidelines publication (document 7) refers to carcinoma *in situ* as including LCIS and DCIS (see page 7) which are category Tis (see page 15, left hand column) which is stage 0, see page 16, Table. In the glossary on page 84 the *in situ* cancers are defined as a very early stage of cancer.

5. Thus the view in the art as set forth by relevant national institutions is that stage 0 breast cancer, which includes DCIS and LCIS, is very early stage breast cancer.

6. Experiments were conducted in Diagenic AS's laboratory to illustrate that different stages of breast cancer may be diagnosed using informative probes isolated from breast cancer patients. In particular we examined whether probes isolated from DCIS (very early stage breast cancer, i.e. stage 0) patients could be used to diagnose patients with stage 0 or I breast cancer using methods of the invention. In these experiments, differentially expressed transcripts were identified in samples from DCIS and IDC breast cancer patients relative to healthy patients and these transcripts were used as probes to predict whether samples were derived from patients with the disease in question or from patients without that disease. The samples which were examined are peripheral blood samples. The experiments which were conducted are described in the following paragraphs.

7. Whole blood from the arms of 48 females were collected in PAX tubes at Ullevål University Hospital Norway. The mean age of females was 58.16 with an age range of 51-82. These included 20 females diagnosed with breast cancer, twenty control females (termed as healthy) who had suspected first mammograms, but were later diagnosed as not having breast cancer, and eight females having another form of cancer, namely colon cancer. Samples from patients with colon cancer were used to show the specificity of the breast cancer probes for diagnosing breast cancer. Among those having breast cancer, ten females had stage 0 cancer (DCIS in which the cancer is confined to the milk ducts) with a tumour size ranging between 4 mm and 50 mm and ten females had stage I cancer (Invasive Ductal carcinoma, IDC) with a tumour size ranging between 2 mm and 19 mm. Details of the breast cancer patients used in the study are provided in Table 1, attached in Annex 1.

8. Total RNA was extracted from the blood samples and subjected to reverse transcription to yield first strand cDNA from which DIG-cRNA probes were prepared by amplification. The probes were hybridized to high density arrays (Applied Biosystems Human Genome Survey Microarray v2.0) containing 32,878 oligonucleotide probes. The amount of labelled probes binding to the immobilized oligonucleotides was assessed and quantified using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer.

9. The generated expression data were then processed. The data were normalized to

take account of the differences in the probe intensities resulting from the experimental conditions. The data set was then analyzed to identify differentially expressed informative probes for different sample groups. Informative probes were identified for the following set ups:

Set up 1: DCIS and IDC vs Healthy samples.
Set up 2: DCIS vs Healthy samples.
Set up 3: IDC vs Healthy samples.
Set up 4: Colon cancer vs Healthy samples.

10. Several genes were found to be informative in the different set ups. Table 2 (attached in Annex 2) provides a matrix of informative genes in each set up and the number of informative genes that were found overlapping among the different set ups. Thus for example in the case of set up 1, 197 genes were identified as informative and reflect differential expression between DCIS and IDC samples vs healthy samples. Of these 197, 133 were also found to be informative in set up 2, i.e. for discrimination between DCIS and healthy samples.

11. To illustrate the utility of these informative probes diagnostically, classification models were generated. The classification models were able to classify individuals from different classes into distinct groups e.g. DCIS from healthy samples (data not shown). The ability of the general model to correctly diagnose samples was determined by cross-validation in which the step of generating the classification model was performed by omitting the data of a single sample from the data used in that modelling process. This process was repeated for each sample to obtain information on the prediction accuracy of the calibration model.

12. The prediction result of the classification model for the 4 set ups is shown in Figures 1A-1D (attached in Annex 3). In the 4 prediction plots, the diseased samples appear on the x axis at +1 and the healthy samples appear at -1. The y axis represents the predicted class membership. During prediction, if the predicted class is correct, the diseased samples should fall above zero and the healthy samples should fall below zero. Correct prediction was achieved for all samples in set ups 2-4. In set up 1 only a small number of samples were incorrectly predicted.

13. This illustrates that breast cancer samples can be readily discriminated from normal samples using the identified probes (Figures 1A-1C). Although not according to the invention as now claimed, for comparative purposes, Figure 1D shows that colon cancer samples can be readily discriminated from normal samples using probes identified when comparing normal and colon cancer samples. Of particular relevance, Figure 1B shows that probes which are informative for DCIS (i.e. very early stage cancer, namely stage 0) relative to healthy patients can be identified. These results show that blood cells from stage 0 breast cancer patients exhibit characteristic changes in their gene expression pattern and this pattern can be used diagnostically for early detection of breast cancers.

14. The ability of the built-in cross-validated models to predict the class of a sample group that was <u>not</u> included during model-building process (test set prediction) is presented in Figures 2A - 2D. In these prediction results, samples below 0 are classified as healthy and samples above 0 are classified in the diseased group.

15. The set up 1 probes which are able to distinguish between DCIS and IDC and healthy samples were used to predict the class of colon cancer samples. It will be seen from the results in Figure 2A, that the cross-validated model correctly predicted the class of 6/8 colon cancer samples as non-breast cancer, i.e. below 0. This result shows that the identified probes for breast cancer are specific and can efficiently discriminate breast cancer from other forms of malignancies.

16. The results in Figure 2B show that probes identified as being informative for discriminating between DCIS and healthy samples can also be used to predict the category of IDC (stage 1) patients as breast cancer samples. In the model 8/10 IDC samples were correctly predicted.

17. In the case of set up 3, the built-in cross-validated model based on informative probes which discriminated between IDC and healthy samples correctly predicted the class of 8/10 DCIS samples (Figure 2C). This illustrates that the altered expression in the IDC samples reflects at least some alterations also seen in DCIS samples. Thus this provides evidence that the cells being examined are cells which are not cancer cells and have not been in direct contact with the disease area.

18. In the case of set up 4, the built-in model correctly predicted the class of 19/20 breast cancer samples as non-colon cancer (Figure 2D). The result shows that these samples were effectively predicted as "healthy" samples, i.e. the informative probes are able to distinguish between different types of cancers.

19. In summary the results show that probes can be identified from blood cells of very early stage (stage 0) breast cancer patients and that these probes can be used to diagnose breast cancer patients with stage 0 or later stage breast cancer. The probes are specific for breast cancer diagnosis and are able to distinguish between breast cancer patients and patients with a different cancer, in this case, colon cancer. These methods of the invention allow screening of patients to easily and rapidly identify those with breast cancer, even those with very early stage (stage 0) breast cancer. Such rapid, cost-effective and non-invasive methods offer considerable advantages over the invasive, time-consuming and painful methods of breast cancer diagnosis that were available prior to the present invention.

20. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such wilful false statements may jeopardize the validity of the application and any patent issuing thereon.

Praveen Sharma ..... 20th September, 2007

Praveen Sharma ..... Date

ANNEX 1

Table. 1: Details of the breast cancer patients used in the study

Stage 0 - DCIS

Sample number	Age	Breast cancer subtype	Histology
1	70	DCIS	25mm
2	64	DCIS	4mm
3	62	DCIS	5mm
4	65	DCIS	30mm
5	59	DCIS	30mm
6	65	DCIS	20mm
7	51	DCIS	12mm
8	na	DCIS	26mm
9	na	DCIS	2mm
10	na	DCIS	50mm

Stage I - IDC

Sample number	Age	Breast cancer subtype	Histology
11	58	IDC	4mm
12	69	IDC	17mm
13	51	IDC	15mm
14	57	IDC	8mm
15	57	IDC	12mm
16	68	IDC	19mm
17	64	IDC	9mm
18	53	IDC	<20mm
19	60	IDC	7mm
20	68	IDC	15mm

ANNEX 2

Table 2. Matrix of differentially expressed informative genes in the different setups. H: healthy. Breast cancer samples includes both stage 0 (DCIS) and stage I (IDC) samples.

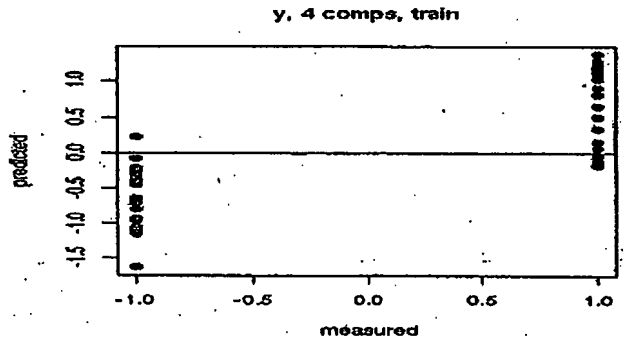
	Set up 1 (breast cancer/H)	Set up 2 (DCIS/H)	Set up 3 (IDC/ H)	Set up 4 (CC/H)
Set up 1 (breast cancer/H)	197	133	106	17
Set up 1 (DCIS/H)		647	88	37
Set up 3 (IDC/ H)			494	36
Set up 4 (CC/H)				735

ANNEX 3

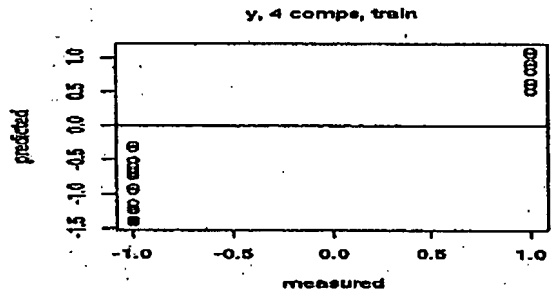
FIGURE 1

Prediction plots (Breast cancer study)

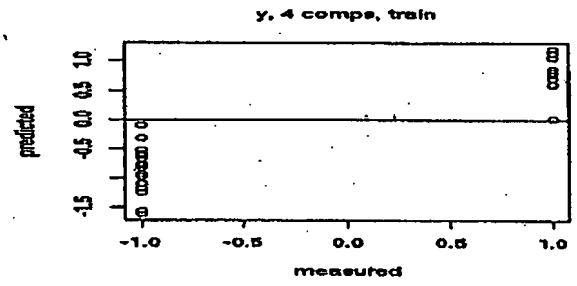
A **Set up 1**



B **Set up 2**



C **Set up 3**



D **Set up 4**

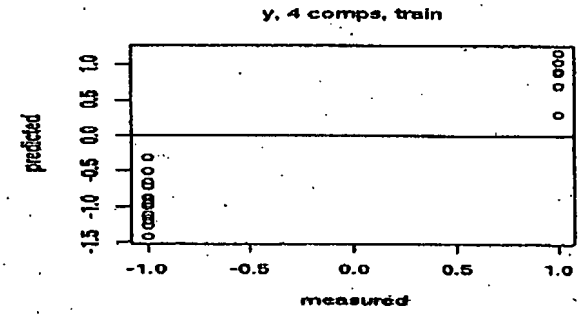


Figure 1. Prediction plot based on informative genes identified in the four different set ups. **1A:** set up 1, breast cancer (both DCIS and IDC) versus healthy samples. **1B:** set up 2, DCIS versus healthy samples. **1C:** set up 3, IDC versus healthy samples. **1D:** set up 4, colon cancer versus healthy samples. Healthy samples appear on the x-axis at -1 and diseased samples appear at +1;

FIGURE 2

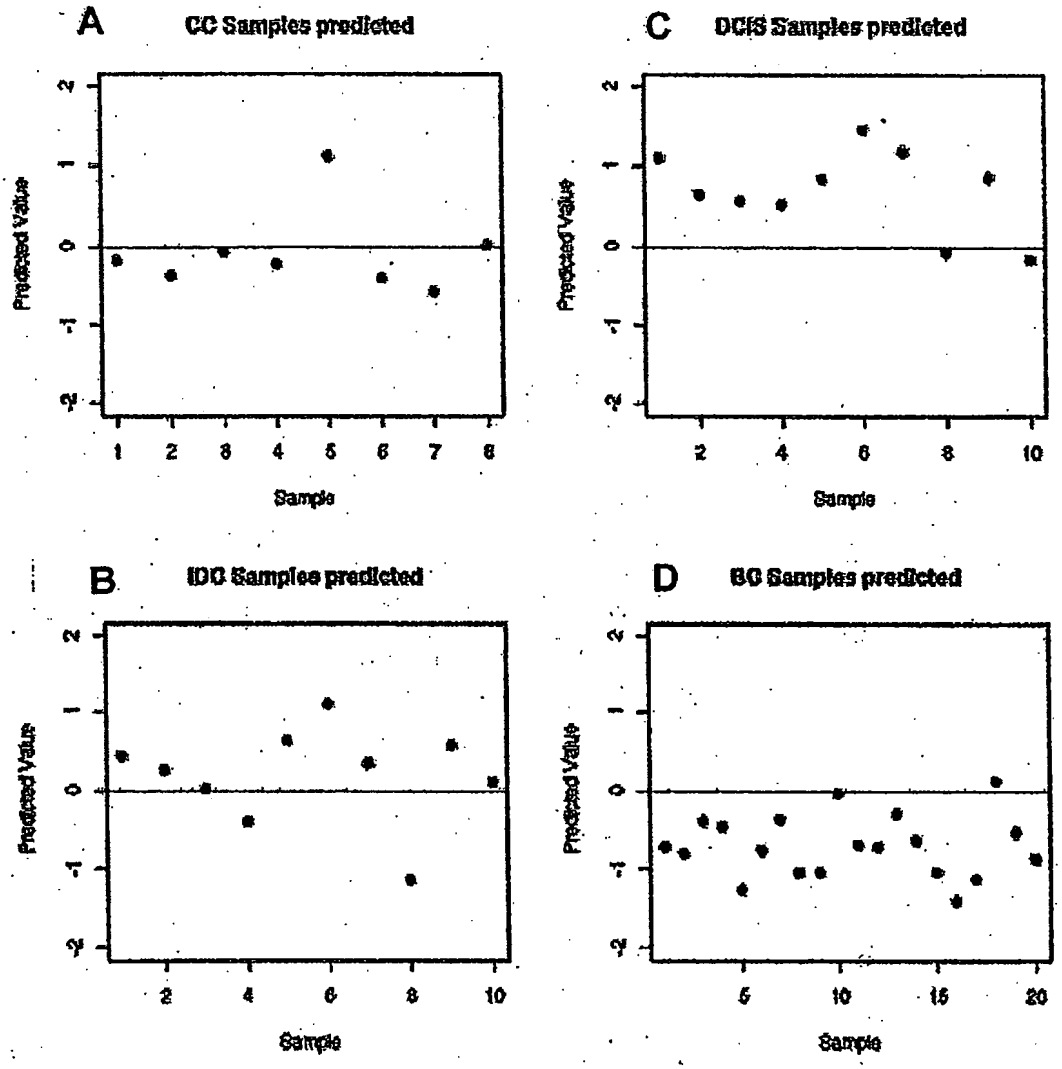


Figure 2. Prediction results. 2**A:** Prediction of colon cancer samples by the model based on breast cancer and healthy samples. **2B:** Prediction of IDC samples by model based on DCIS and healthy samples. 2**C:** Prediction of DCIS samples by model based on IDC and healthy samples. 2**D:** Prediction of breast cancer samples by model based on colon cancer and healthy samples.

ANNEX 4